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The Application of Self-Assembling Nanoemulsion (Sane) in the Delivery of the Antibiotic Cefazolin

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Abstract

Antimicrobial resistance is an important health issue. Recent developments in nanotechnology have made possible opportunities to develop novel delivery options for pharmaceuticals and other biological agents already on the market today. In this study, an oil-in-water nanoemulsion and an aqueous solution of Cefazolin at 30 ug/mL and serial dilutions thereof, were tested against a quality control strain of *Staphylococcus aureus (S. aureus*). A *S. aureus* bacterial suspension was streaked on to nutrient agar (NA) plates. Cefazolin preparations were added to wells aseptically bored out at 5 drops/well. The observed inhibitory zones were measured in millimeters. Our results demonstrated that the Cefazolin nanoemulsion formulation and Cefazolin aqueous solution at 30 ug/mL produced an inhibitory effect of 23 and 24 mm respectively. Quantitative plate counts of the Cefazolin preparations were also evaluated. Serial dilutions of both Cefazolin preparations were made in a 96- well microtiter plate using Mueller Hinton Broth (MHB). A suspension of *Staphylococcus* aureus equal to 1.0×10^7, was added to the wells and this plate was incubated at 37 °C for 16-18 hr. Post incubation, the wells were subbed to NA plates and examined for CFU/mL. The NA plates containing the Cefazolin nanoemulsion formulation produced a decreased CFU/mL compared to the Cefazolin aqueous solution. Accordingly, the results of this study suggest that by decreasing the concentration of an antibiotic by its incorporation into a nanoemulsion formulation, we may reduce the amount of antibiotic needed to be delivered to produce both an inhibitory effect and a decrease in CFU/mL, thus reducing the potential for increased incidence of antibiotic resistant infections.

Keywords: Cefazolin; Nanoemulsion; Antimicrobial Resistance; Well Diffusion; Agar Diffusion; Inhibition; Bactericidal

Abbreviations: CDC: Centers for Disease Control and Prevention; MRSA: Methicillin-Resistant *Staphlococcus Aureus;* VRE: Vancomycin-Resistant *Enterococci;* KPC: *Klebsiella Pneumoniae* Carbapenemase; *S. aureus: Staphylococcus aureus;* HAIs: Hospital-Acquired Infections; SANE: Self-Assembling Nanoemulsion; NA: Nutrient Agar.

Introduction

Antimicrobial resistance is a global problem and continues to be a challenge for the healthcare industry as more resistant strains of bacteria develop [1-3]. The Centers for Disease Control and Prevention (CDC) considers this altered resistance a threat to public health. According to CDC statistics, more than 2 million people develop bacterial-resistant infections each year and according to the 2013 Threat Report, at least 23,000 people died as a result [3]. Subsequently, many others die from complications that arise from these antibiotic-resistant infections. Methicillin-resistant *Staphlococcus aureus (*MRSA) and Vancomycin-resistant *Enterococci* (VRE) are two prime examples of antibiotic-resistant bacteria. *Klebsiella pneumoniae* carbapenemase (KPC) infections, once seen in limited locations in the U.S., are now found throughout the country [3]. *Clostridium difficile* infections, while not resistant to the medications that are used to treat them, are directly related to antibiotic use [3]. Patients that become infected with antibiotic-resistant micro-organisms require extended hospital stays involving intensive treatment with additional antibiotics that are ultimately less effective, toxic, and more expensive [1-3]. Consequently, treatment of resistant infections results in US healthcare costs in excess of $20 billion per year [3].

Despite the number of antibiotics available that treat infection, bacteria have adapted to overcome their effects through spontaneous mutation and the acquisition of resistance genes. *Staphylococcus aureus* (*S. aureus*) is one of the most commonly isolated pathogens and is responsible for a number of infections [4]. It is also the leading cause of hospital-acquired infections (HAIs) in the U.S. [4,5]. Current CDC statistics reveal that MRSA is responsible for at least 11,000 U.S. deaths and 80,000 invasive infections per year [3]. MRSA infections, once thought to be common among hospitalized patients, are now becoming commonplace in the community and have created a public health crisis [6].

In an effort to combat increasing microbial resistance, novel therapies have emerged. Nanoemulsion use is rapidly becoming a delivery option for pharmaceuticals and other biological agents [7-10]. Such nanoformulations are: emulsions, which are oil-in-water (O/W) mixtures; suspensions, which are surfactant/bioactive/water combinations; and liposomes, which can also be bioactives and water sandwiched between a surfactant phospholipid bilayer. These nanoformulations have particle sizes around 10^{-9} and can be made by simple mixing or through the use of high mechanical stress to change larger droplets into smaller ones [7-10]. Addition of the appropriate type and amount of surfactant can extend the integrity of the nanoformulation for years [7-10]. When pharmaceuticals and bioactives are added to an emulsion system, it results in an increased surface size and bioavailability [7-10]. This technology has shown to be an effective delivery system with both biological materials and pharmaceuticals [7-10]. Evidence of successful nanoemulsion formulae from this laboratory include nanoemulsion preparations of the lipid soluble breast cancer drug, Tamoxifen, which has been shown to decrease cell proliferation in breast cancer cell lines [11]. Self-assembling nanoemulsion (SANE) preparations of the anti-cancer drug, Paclitaxel, have been shown to inhibit cell proliferation in breast and pancreatic cancer cell lines [12]. SANEs of the cholesterol-lowering statin drug, Lovastatin, have been shown to reduce cholesterol and lipoprotein accumulation [13]. Nanoemulsions of Dextran have been shown to increase cell density in culture better than micro carriers of Dextran [14]. Nanoemulsions of the anti-cancer/anti-melanoma drug, Dacarbazine, when administered intramuscularly or topically, show decreased tumor size when compared to a Dacarbazine suspension [15,16]. Nanoemulsions of the carotenoid, lutein, show greater bioavailability than lutein supplements [17].

Cefazolin is a first generation Cephalosporin antibiotic used to treat *Staphylococcus*, *Streptococcus* and select gram-negative infections. It functions by inhibiting bacterial cell wall synthesis. SANEs are oil-in-water (O/W) mixtures of small particle size capable of encapsulating many molecules, increasing their surface size and rendering them readily bioavailable. A SANE preparation of Cefazolin was used to determine if an antibiotic prepared as a nanoemulsion was able to inhibit bacterial growth greater and at a reduced concentration than an aqueous solution of Cefazolin.

Materials and Methods

Media and Microorganism Preparation

Susceptibility testing was performed using Nutrient agar [NA] plates that were purchased from Becton

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Dickinson and Company [Sparks, MD] and prepared according to manufacturer instructions. All plates were poured and allowed to cure for 24 hr at room temperature. Lyophilized *Staphylococcus* aureus *subsp*. aureus ATCC® #25923™ [ATCC®, Manassas, VA] was reconstituted according to supplier's specifications and sub cultured daily to a NA plate to maintain organism viability. The subculture used in experimental methods was prepared as a frozen stock. Briefly: 5-10 well isolated colonies of a pure culture were sub-cultured in a sterile 15 mL conical tube in Mueller Hinton Broth [MHB] [Northeast Laboratory, Waterville, ME]. The culture was incubated at 37°C on a rocker until mid-log phase of growth [~2 hr]. The culture was then adjusted spectrophotometrically to 0.5 optical density [OD] at 600 nm with sterile MHB. The adjusted culture was mixed 1:1 with 40% glycerol, vortexed for 2 min and aliquoted to 1 mL sterile cryovials. Aliquots were frozen at -80°C until use. Colony forming unit concentration/mL [CFU/mL] of the subculture stock was 1.0 x 10^7.

Cefazolin Nanoemulsion and Cefazolin Aqueous Solution Preparation

Cefazolin [Sigma Aldrich, St. Louis, MO] nanoemulsions were prepared by O/W emulsification through self-assembly [SANE] and phase-inversion technology [PIT] using Cefazolin sodium salt resulting in a concentration of 30 ug/mL. The Cefazolin nanoemulsion was made by adding one gram of rice bran oil [Tsuno, Japan] to a beaker and heated slowly. 1.5 mg of Cefazolin was added to the oil and this mixture was heated and stirred with a magnetic stirrer until the Cefazolin dissolved completely at 50-60°C. Five grams of the surfactant Solutol HS-15 [BASF, Germany] was added to this mixture, heated and stirred for additional 5 minutes at 50-60°C until a homogeneous mixture was formed. Forty-four mL of Nano pure water was added to bring the total volume of Cefazolin nanoemulsion to 50 mL while the mixture was stirred at 60°C. During heating when the phase inversion temperature of the system is reached [65-70°C; the phase inversion zone] the surfactant is in equilibrium with the oil and water phases. Heating and stirring was continued beyond the phase inversion temperature to 80°C. At this point the system converts to a water-in-oil [W/O] emulsion. Once the system is cooled to room temperature, it forms an oil-in-water [O/W] emulsion. A blank nanoemulsion was prepared with the same ingredients as the Cefazolin nanoemulsion, but without the Cefazolin. The mean droplet size, width and poly-dispersity index [PdI] was measured by a Malvern Zetasizer Nano instrument [Malvern Instruments, Inc., Southborough, MA]. Cefazolin aqueous solution was prepared at the same concentration as the Cefazolin nanoemulsion of 30 ug/mL [total volume of suspension = 50 mL] with nano-pure water as the solvent.

Susceptibility Testing Using Well Diffusion

The *S. aureus* bacterial suspension was streaked to a NA plate covering the entire sterile agar surface in three directions to ensure an even distribution of inoculum. Wells were aseptically bored out of the inoculated NA. Cefazolin nanoemulsion and Cefazolin aqueous solution at the concentration previously described above, were added at 5 drops/well, equal to approximately 150 uL. Each Cefazolin preparation was run in triplicate. An additional NA plate streaked with the *S. aureus* suspension, had a well bored out that contained 5 drops of the blank nanoemulsion. All plates were incubated at 37°C for 16-18 hr. Wells were observed for inhibitory zones and measured in millimeters.

Toxicity of the Nanoemulsion Surfactant

Additional NA plates were prepared that included the blank nanoemulsion [that contains all of the nanoemulsion ingredients without the antibiotic Cefazolin] in different surfactant concentrations of 1%, 5% and 10%. These blank nanoemulsions were incorporated directly into the media at 1 mL of each concentration of surfactant/20 mL of agar. These plates were made to test for toxicity of the surfactant, Solutol HS-15, that was used in the nanoemulsion preparation. The *S. aureus* bacterial suspension was streaked to these plates and the plates were evaluated for the presence or absence of bacterial growth.

Quantitative Plate Counts

Serial dilutions of both 30 ug/mL Cefazolin preparations [dilution range = 15, 7.5, 3.7, 1.8, 0.9 and 0.4 ug/mL] were made in MHB and 100 uL of each dilution series was pipetted in triplicate into a 96-well microtiter plate [Corning Incorporated, Corning, NY]. Ten microliters of *S. aureus* equal to 1.0 x 10^7, was added to the wells and the microtiter plate was incubated at 37 °C for 16-18 hr. Post incubation, a 10 uL sample from the well with the highest optical density [OD] of each dilution series of Cefazolin nanoemulsion and Cefazolin aqueous solution was sub cultured to a NA plate and evaluated for CFU/mL. Agar plates were run in duplicate.

Cefazolin Encapsulation by High Pressure Liquid Chromatography [HPLC]

Aliquots of the Cefazolin nanoemulsion were spun in Eppendorf tubes at 18,000 rcf for 15 minutes. Ten

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microliter of supernatant was diluted in 40 uL of Phosphate Buffered Saline (PBS) and un-encapsulated Cefazolin was determined. Ten microliter aliquots of Cefazolin nanoemulsion pellet were diluted in 40 uL PBS and encapsulated Cefazolin was determined. The chromatographic method was performed on an Agilent 1100 Series system, consisting of quaternary pump and a DAD UV detector. The column was a C18 Phenomenex Kinetex [100 x 4.6mm] with a particle size of 2.6 um as a stationery phase. The mobile phase consisted of a 4:1 mixture of ammonium formate [20uM] plus 100% acetonitrile pumped into the column at a flow rate of .5 mL/minute at room temperature. The detector was monitored at 200 - 400nm and the run time was 6.0 minutes. The injection volume was 10 uL. ChemStation software [Agilent Technologies, Santa Clara, CA] was used for analysis.

Statistical Measurements

Statistical measurements were performed using IBM® Statistical Package for the Social Sciences [SPSS®] version 25. After frequencies were calculated for each condition, Fishers Exact test was used to calculate significance. Statistical significance was achieved at p = <.001.

Results

Characteristics of the Cefazolin Nanoemulsion

It was important to evaluate the toxicity of Solutol HS-15, including the blank nanoemulsion components, before the preparation of SANEs. The NA plates that were made with Solutol HS-15 added to the media in concentrations of 1%, 5% and 10% were used to check for surfactant toxicity to the bacterial organism. Uniform bacterial growth was seen on all plates in all concentrations of surfactant (Figure 1). No quantitation of colonies was performed as any bacterial growth that occurred in the presence of the surfactant indicated that the surfactant was non-toxic. Nanoemulsion preparation continued using this surfactant at the concentration of 5%.

Figure 1: Solutol Toxicity Plates. Solutol HS-15 shows no toxicity to S. aureus at concentrations of 1%, 5% and 10% respectively

A schematic diagram of the nanoemulsion particle can be seen in Figure 2. The Cefazolin is contained within the oil core surrounded by the surfactant. The surfactant provides decreased surface tension by utilizing both a hydrophilic head and hydrophobic tail that is needed for the water and oil to remain in solution and for the emulsion to remain stable. The particle size of the nanoemulsion was 18.8 nm and was not significantly influenced by concentration of antibiotic. The polydispersity index [Pdl] was 0.048 (Figure 3).

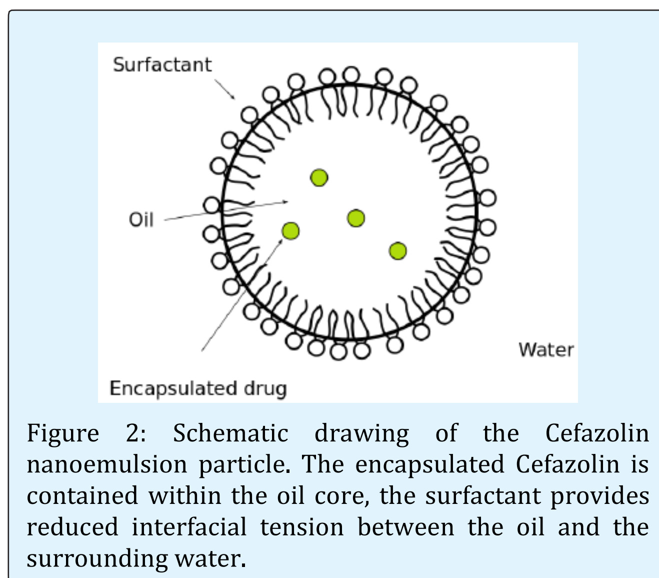

Figure 2: Schematic drawing of the Cefazolin nanoemulsion particle. The encapsulated Cefazolin is contained within the oil core, the surfactant provides reduced interfacial tension between the oil and the surrounding water.

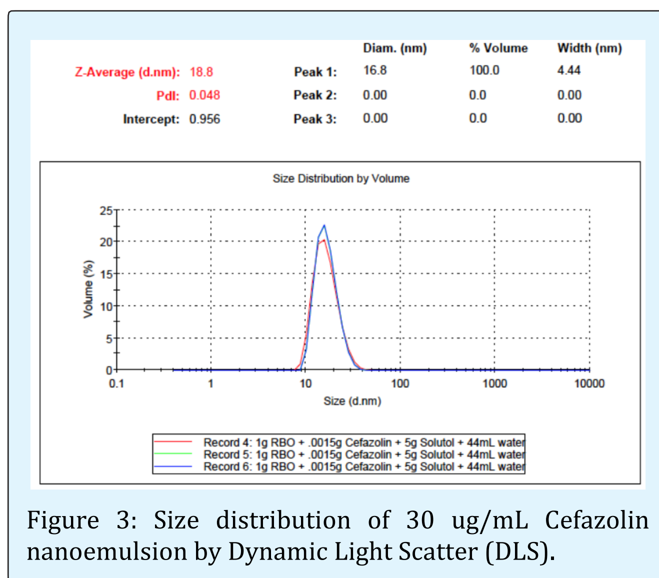

Figure 3: Size distribution of 30 ug/mL Cefazolin nanoemulsion by Dynamic Light Scatter (DLS).

Encapsulation Efficiency of the Cefazolin Nanoemulsion

Approximately 56% of Cefazolin was encapsulated [13.6 ug] in the nanoemulsion vs. 44% [10.8 ug] that was

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un-encapsulated. Encapsulation efficiency of the nanoemulsion was 45.3%.

Inhibitory Activity of the Cefazolin Nanoemulsion

The Cefazolin nanoemulsion was inhibitory to *S. aureus*, evidenced by the 23 mm [mean = 23, SD = 1] zone of inhibition present at 30 ug/mL (Figure 4). Likewise, the Cefazolin aqueous solution was inhibitory at 24 mm [mean = 24, SD = 1] to *S. aureus* at 30ug/mL as well (Figure 4). Components of the nanoemulsion were not inhibitory to *S. aureus* (Figure 4).

Figure 4: Zone of inhibition measurements of Cefazolin nanoemulsion and Cefazolin aqueous solution in comparison to the nanoblank. Components of the nanoemulsion do not inhibit the growth of *S aureus* (on left). Cefazolin nanoemulsion at 23 mm (n = 2) is inhibitory to *S. aureus*; Cefazolin aqueous solution at 24 mm (n = 2) is inhibitory to *S. aureus* (on right).

Quantitative Plate Counts [Spread Plate Technique]

Plate counting criteria was as follows: plates that contained less than 30 and greater than 300 colonies were not counted. Plates that contained at least 30 but not more than 300 colonies were counted. Plates that were included in the countable category were plates that contained the dilutions of nano and aqueous Cefazolin at 15 and 7.5 ug/mL (Figure 5). Cefazolin nanoemulsion at 15 ug/mL yielded 91.7% countable NA plates [30-300 colonies] and 8.3% uncountable NA plates [>300 colonies] vs. the Cefazolin aqueous solution that yielded 7.1% countable NA plates and 92.9% uncountable NA plates [Figure 6]. Likewise, at 7.5 ug/mL, Cefazolin nanoemulsion yielded 78.6% countable NA plates and 21.4% uncountable NA plates, vs. 100% of Cefazolin aqueous solution NA plates that were uncountable [Figure 7]. Nutrient agar plates containing the dilutions of 3.75 – 0.4 ug/mL did not meet the countable colony criteria. These dilutions produced plates with bacterial populations that were overgrown.

Figure 5: NA plates of CFU/mL of Cefazolin nanoemulsion vs. Cefazolin aqueous solution. Cefazolin nanoemulsion (bottom row) yields lower colony counts of *S. aureus* than Cefazolin aqueous solution (top row) at concentrations of 15 ug/mL, 7.5 ug/mL respectively.

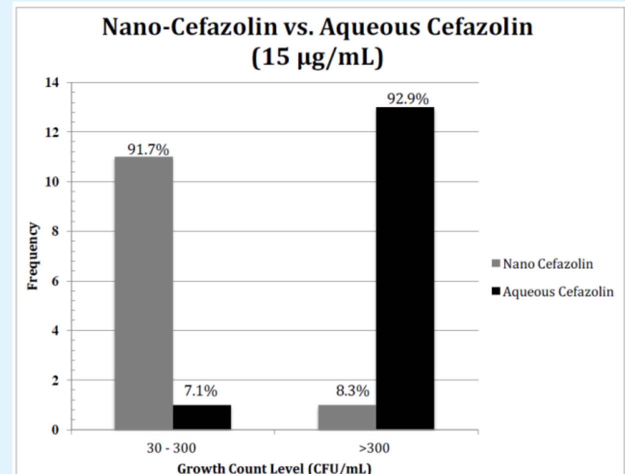

Figure 6: Quantitative plate count data of Cefazolin nanoemulsion vs. Cefazolin aqueous solution at 15 ug/mL. Exposure of *S. aureus* to Cefazolin nanoemulsion at 15 ug/mL results in lower colony counts than Cefazolin aqueous solution. P = <.001

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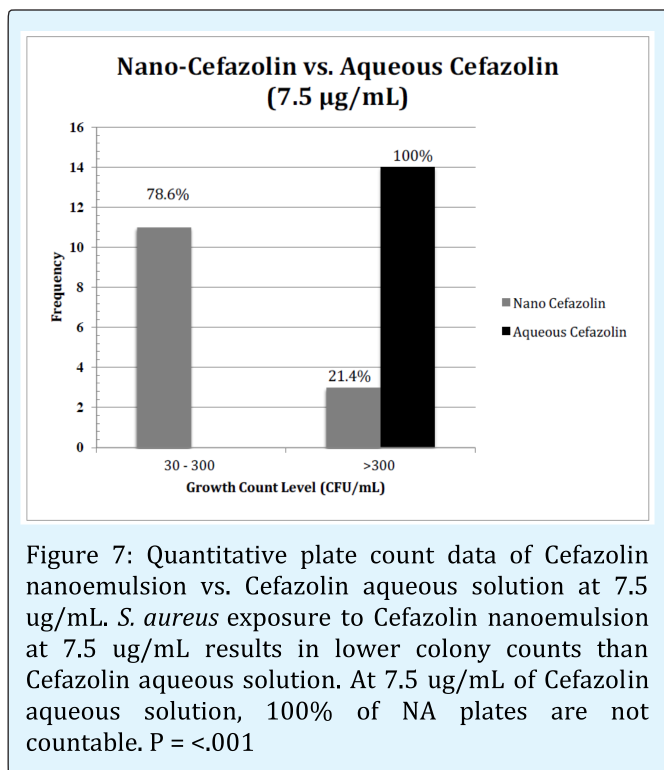

Figure 7: Quantitative plate count data of Cefazolin nanoemulsion vs. Cefazolin aqueous solution at 7.5 ug/mL. *S. aureus* exposure to Cefazolin nanoemulsion at 7.5 ug/mL results in lower colony counts than Cefazolin aqueous solution. At 7.5 ug/mL of Cefazolin aqueous solution, 100% of NA plates are not countable. P = <.001

Discussion

We utilized *Staphylococcus aureus subsp. aureus* ATCC® #25923™ a methicillin sensitive quality control strain that has many applications in the clinical microbiology lab. We used SANE technology for this study because of its successful use in previous experiments in our laboratory [11- 17]. We wanted to determine if SANE would be an acceptable method to encapsulate Cefazolin, reducing the amount of antibiotic delivered to produce the same effect as an aqueous form of Cefazolin. *S. aureus* was chosen for this study because this organism has become resistant to many antibiotics currently used for treatment. Cefazolin is a first generation Cephalosporin antibiotic that is used to treat *Staphylococcus* infections but it is not effective against MRSA. However, *S. aureus* is an important pathogen on its own as it is the etiologic agent of many infections, including: bacteremia, pneumonia, osteomyelitis, endocarditis, toxic shock syndrome, boils, cellulitis, impetigo and food poisoning [18- 20].

Comparison of well diffusion between each preparation shows the aqueous solution of Cefazolin at 30 ug/mL [24 mm] resulted in a zone of inhibition that was greater than the Cefazolin nanoemulsion [23 mm]. We feel that the slight increase in diffusion of the Cefazolin aqueous solution is due to the fact that the Cefazolin nanoemulsion did not diffuse into the agar as efficiently as the Cefazolin aqueous solution and this diffusion could be influenced by the density of the oil and the surfactant. Numerous attempts to measure the concentration of both nano and aqueous Cefazolin diffusing from the wells resulted in poor recovery of Cefazolin from the NA. Agar plugs of both nano and aqueous Cefazolin removed at various time points around the diffusion wells yielded undetectable levels of Cefazolin. However, washouts of the diffusion wells of the Cefazolin nanoemulsion and Cefazolin aqueous solution, once the Cefazolin nanoemulsion [2 hr.] and the Cefazolin aqueous solution [1hr.] had completely diffused from the wells, revealed that 99% of both preparations had diffused from the well [data not shown]. In contrast, when evaluating an antibiotic to treat a clinical infection, choosing an antibiotic with the largest zone size is not always appropriate. Comparative to Minimum Inhibitory Concentrations [MIC], specific numerical interpretations do not show which drugs are most effective. There are many issues to consider, including the location of an infection, mode of antibiotic delivery, status of the patient and the inherent toxicity of certain drugs.

A count of CFU/mL between Cefazolin preparations was not performed, due to the amount of uncountable plates seen with the Cefazolin aqueous solution preparations. It is clear from the data presented, that the nanoemulsion is inhibiting *S. aureus* better than the Cefazolin aqueous solution because of the decreased number of CFU on the Cefazolin nanoemulsion NA plates at 15 and 7.5 ug/mL. The decrease in *S. aureus* colonies that is seen with the Cefazolin nanoemulsion is presumably due to the small particle size [18.8 nm], the larger amount of Cefazolin that is contained within the nanoemulsion [56%], and the increased bioavailability, efficacy and stability of Cefazolin that the nanoemulsion system provides [11-17]. We observed a loss of Cefazolin [5.6 ug] during the encapsulation process but this was expected. Previous experience with alternative encapsulation procedures [microfluidization] resulted in some sample loss in our formulations. Regardless of the loss, the effect of the encapsulated Cefazolin was favorable.

Overgrowth of bacteria on NA plates of the Cefazolin nanoemulsion seen at the concentrations of 3.75 – 0.4 ug/mL was disappointing, as our goal was to observe a measurable difference in the Cefazolin nanoemulsion at the sub-MIC level of Cefazolin [< 0.2 ug/mL]. We plan to continue Cefazolin formulations in additional

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nanoparticles in order to achieve sub-MIC inhibition of *S. aureus*.

Conclusions

This study suggests that decreasing the concentration of Cefazolin by its incorporation into a nanoemulsion, results in greater bioavailability and efficacy at inhibiting *S. aureus*. Additionally, CFU/mL of *S. aureus* was reduced by Cefazolin nanoemulsion. SANE delivery of an antibiotic has the potential to deliver decreased dosages capable of altering the environment that favors resistance.

Conflict of Interest

The authors declare that they have no conflict of interest in the publication.

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